Method for Selectively Isolating a Nucleic Acid

ABSTRACT

Provided are methods for selectively identifying and isolating nucleic acids in a population of nucleic acid molecules.

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Related Application

This application claims priority to USSN _____, filed December 8, 2000, and USSN 60/170,140, filed December 10, 1999, which incorporated herein by reference in their entireties.

Field of the Invention

The invention relates to compositions and methods for identifying nucleic acids in a population of nucleic acids

Background of the Invention

One major area of current clinical research is the correlation of an individual's genetic profile to a susceptibility to disease and/or response to drug therapy. This area of research, which has been labeled pharmacogenomics, offers a strategy for targeting drugs to individuals, and for elucidating genetic predispositions and risks. In addition, pharmacogenomics provides for the possibility for an improved drug discovery process based on a better understanding of the molecular bases of complex diseases.

Identification of an individual's genetic profile can require the identification of particular nucleic acid sequences in the individual's genome. These particular nucleic acid sequences can include those that differ by one or a few nucleotides among individuals in the same species. For example, single-nucleotide polymorphisms (SNPs) are common variations in the DNA of individuals that are used to track inherited genetic patterns [1].

Current methods for identifying nucleic acid polymorphisms can be labor-intensive and expensive.

Summary of the Invention

The invention is based in part on the discovery of a method for rapidly and economically isolating nucleic acid sequences containing particular nucleic acid sequences of interest. The

invention provides a composition and method for sequence-specific extraction of polynucleotide sequences from a potentially complex mixture of nucleic acids. One method of the invention, which is termed 'Allele-Specific Extraction' (ASE), enables the distinction of two nearly identical sequences, for instance genes of maternal and paternal origin, by physical separation based on the identity of a heterozygous site. This ability, when coupled with standard methods commonly used for genotyping, permits rapid large-scale and cost-effective haplotyping of individuals, which can significantly reduce the size and decrease the duration of genetic profiling studies by focussing on the analysis of rare events, such as therapeutic non-responders or adversely affected individuals [2].

In one aspect, the invention provides a method for separating a nucleic acid of interest from a population of nucleic acid molecules. The method includes providing a population of nucleic acid molecules, contacting the population of nucleic acid molecules with a first targeting element, wherein the first targeting element binds specifically to at least one nucleic acid sequence of interest in the population of nucleic acid molecules, and attaching (or removing) a separation group to the targeting element. The attached separation group is then immobilized on a substrate, thereby forming an immobilized targeting element-separation group-nucleic acid sequence complex. The immobilized targeting element-separation group complex is then removed from the population of nucleic acid molecules, thereby separating the nucleic acid sequence of interest from the population of nucleic acid molecules.

In general, any population of nucleic acids can be used in the method. For example, polynucleotide sequences can be, *e.g.*, DNA or RNA, and can include genomic DNA, plasmid DNA, amplified DNA, cDNA, total cellular RNA, hnRNA, polyA+-containing RNA. Nucleic acids can be from a single unicellular or eukaryotic organism. For example, the nucleic acid can be obtained from a mammalian organism such as a human.

If desired, the population of nucleic acids can be amplified using PCR or another amplification technique for the fragment(s) of interest prior to performing allele-specific extraction if the amount of available starting nucleic acid insufficient for direct separation and subsequent analysis

The targeting element is a molecule that binds specifically to a nucleic acid sequence in a population of nucleic acid molecules. In some embodiments, the targeting element is a nucleic acid, or nucleic acid derivative that hybridizes to a complementary target sequence in a population of nucleic acids. Examples of nucleic acid-based nucleic acid derivatives include,

e.g., an oligonucleotide, oligo-peptide nucleic acid (PNA), oligo-LNA, or a ribozyme. The targeting element can alternatively be a polypeptide or polypeptide complex that binds specifically to a target sequence. Examples of polypeptide-based target elements include, *e.g.*, a restriction enzyme, a transcription factor, RecA, nuclease, any sequence-specific DNA-binding protein. The targeting element can alternatively, or in addition be a hybrid, complex or tethered combination of one or more of these targeting elements.

In some embodiments, the targeting element binds to a target nucleic acid sequence in the vicinity of a discrete sequence known as a distinguishing element. A distinguishing element can include any sequence of interest. For example, the distinguishing element can be, *e.g.*, a polymorphism (such as a single nucleotide polymorphism), a restriction site, a methylated restriction site, methylated sequence motif, secondary structure.

Association of a targeting element with a sequence of interest (such as one in the vicinity of a distinguishing element) can occur as part of a discrete chemical or physical association. For example, association can occur as part of, *e.g.*, an enzymatic reaction, chemical reaction, physical association; polymerization, ligation, restriction cutting, cleavage, hybridization, recombination, crosslinking, pH-based cleavage.

The separation group can be any moiety that facilitates subsequent isolation and separation of an attached target element that is itself associated with a target nucleic acid. Preferred separation groups are those which can interact specifically with a cognate ligand. A preferred separation group is an immobilizable nucleotide, *e.g.*, a biotinylated nucleotide or oligonucleotide. For example, separation groups can include biotin. Other examples of separation groups include, *e.g.*, ligands, receptors, antibodies, haptens, enzymes, chemical groups recognizable by antibodies or aptamers.

The separation group can be immobilized on any desired substrate. Examples of desired substrates include, *e.g.*, particles, beads, magnetic beads, optically trapped beads, microtiterplates, glass slides, papers, test strips, gels, other matrices, nitrocellulose, nylon. The substrate includes any binding partner capable of binding or crosslinking identical polynucleotide sequences via the separation element. For example, when the separation element is biotin, the substrate can include streptavidin.

The targeting element preferably includes (in whole or in part) a unique region located in proximity to the distinguishing element. The unique region uniquely identifies a polynucleotide sequence in conjunction with the particular region.

In some embodiments, the targeting element binds to the target nucleic acid sequence immediately adjacent to the distinguishing element. In other embodiments, the targeting element binds to a target nucleic acid sequence with an intervening sequence in between, or partly overlapping with, the distinguishing element.

In various embodiments, the targeting element binds within 100, 50, 20, 15, 10, 8, 7, 6, 4, 3, 2, or 1 , or 0 nucleotides of the distinguishing element.

In preferred embodiments, an enzyme-driven incorporation is performed of a separation element which becomes covalently attached to the targeting element (a specific oligonucleotide). The targeting element can itself be covalently attached or topologically linked to the targeted polynucleotide, which allows washing steps to be performed at very high stringency that result in reduced background and increased specificity.

For example, in preferred embodiments, the oligonucleotide has an extendable 3' hydroxyl terminus. When the targeting element is an oligonucleotide with an extendable 3' hydroxyl terminus and the separation group is an immobilizable nucleotide (such as a biotinylated nucleotide), the separation group is preferably attached to the targeting element by extending the oligonucleotide with a polymerase in the presence of the biotinylated nucleotide, thereby forming an extended oligonucleotide primer containing the immobilizable nucleotide.

If desired, the method can be repeated with second, third, or fourth or additional targeting elements by contacting the population of nucleic acid molecules with an additional targeting element (*e.g.*, a second, third, fourth or more targeting element) that binds specifically to an additional nucleic acid sequence or sequences of interest in the population of nucleic acid molecules (which may be the same or different than the first nucleic acid of interest). A second (or additional) separation group is attached to the second targeting element. The attached second (or additional) separation group is attached to a substrate, thereby forming a second immobilized targeting element-separation group complex. The immobilized targeting element-separation group complex is then removed from the population of nucleic acid molecules, thereby separating the nucleic acid sequence of interest from the population of nucleic acid molecules.

In a further aspect, the invention provides a method for separating a nucleic acid of interest from a population of nucleic acid molecules by providing a population of nucleic acid molecules and contacting the population of nucleic acid molecules with a targeting element attached to a separation group. The targeting element with the attached separation group binds specifically to at least one nucleic acid sequence of interest in the population of nucleic acid

molecules. The separation group is then removed from the bound targeting element. The separation groups are then immobilized to a substrate, thereby forming an immobilized targeting element-separation group complex for at least one nucleic acid sequence. The population of immobilized nucleic acid molecules through the targeting element-separation group complex is separated from nucleic acid sequences not containing the attached separation group.

Among the advantages of the invention is that it is directly compatible with standard genotyping methods and can be easily adapted for multiplexing. In addition, the method can be practiced in a bulk material and does not require single molecule dilution to achieve allele-specific separation. The method can be practiced as a single molecule technique, and the overall speed of the method is expected to be orders of magnitude faster than currently available processes. Moreover, the method does not involve live organisms such as rodents or yeast and thus eliminates any considerations and sources for error associated with such use. In addition, the method is suitable for robotic automation using commercially existing instrumentation for DNA extraction and purification. Moreover, the method allows for the allele-specific analysis of very long fragments of DNA.

The method is well-suited to identifying and isolating nucleic acids containing single nucleotide polymorphisms (SNPs). However, the method is not limited to the use of SNPs but also works with other genetic markers (for instance restriction sites, single tandem repeats, microsatellites), potentially including epigenetic patterns such as methylation. The method allows for the correlation of an unlimited number of sites constituting a haplotype *i.e.*, is not limited to pairwise comparison of two selected sites. The method additionally allows for the generation of a re-usable library of genomic DNA. The library can be used to obtain haplotypes of previously untargeted genomic regions by regular genotype analysis without repeated allele-specific extraction.

In various embodiments, the methods disclosed herein are provided for manual operation in kit format, automated high-throughput operation, and/or in miniaturized & integrated format. The methods can be used in, *e.g.*, human diseases, or predispositions to human diseases (including metabolic disease, cancer typing, diagnosis, and prognosis), analysis of organelle DNA (mitochondrial and chloroplast), plant traits, drug discovery, and in evolutionary studies, including tracking of disease evolution.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention

belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present Specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic illustration of a maternal and paternal chromosomal fragment containing several polymorphisms.

FIG. 2 is a schematic illustration showing annealing of oligonucleotides in the region of a polymorphic site.

FIG. 3 is a schematic illustration showing incorporation of an immobilizable nucleotide.

FIG. 4 is a schematic illustration showing annealing of an oligonucleotide having a 3' base mismatch.

FIG. 5 is a schematic illustration showing elongation of an oligonucleotide lacking a mismatch.

FIG. 6 is a schematic illustration showing separation of a targeted fraction using solid support.

FIG. 7 is a graph showing attachment and release events of individual DNA molecules over time to a single bead as observed by a displacement sensor. FIG. 8 is a graph showing attachment events of individual DNA molecules covalently linked to a separation element-bead complex.

FIG. 9 is a schematic illustration of multiple separation elements topologically locking a target fragment to a solid support.

FIG. 10 is a schematic illustration of a first order multiplexing reaction.

FIG. 11 is a schematic illustration of a second order multiplexing reaction.

DETAILED DESCRIPTION OF THE INVENTION

The method provides for identifying and isolating specific nucleotide sequences in a population of nucleic acids. The method allows for haplotyping through specific chromosomal fragment capture.

In one embodiment, the method is divided into three steps:

1) "Targeting"

In a first step, a targeting element uniquely distinguishing a particular polynucleotide sequence is targeted. FIG. 2 is a schematic illustration showing annealing of oligonucleotides to a polymorphic site.

2) "Distinction"

In a second step, a process is carried out that distinguishes, based on the nature of the distinguishing element, between the targeted polynucleotide sequence(s) and any other sequence(s) present in the material by conditionally attaching or removing a functional group that can serve as a separation element for physical manipulation of the targeted polynucleotide sequence (FIG. 3).

3) "Separation"

In a third step, the targeted polynucleotide sequences are physically separated from the remainder of the sequences in a washing step after selective immobilization to a solid support via the attached separation element.

In an exemplary embodiment, the method allows for separation of DNA fragments of maternal and paternal origin so that differences between the fragments can be assessed for the determination of a haplotype. The method can be practiced by manual operation and standard molecular biological equipment and materials as described below.

If the sample is a combination of alleles from a heterozygous individual, there will be - by definition - locations that distinguish fragments containing the two different alleles. FIG. 1 is a schematic illustration of a maternal and paternal chromosomal fragment containing several polymorphisms, including heterozygous polymorphisms.

The steps are described in more detail below.

1) Targeting

This step results in the recognition of a region within a polynucleotide sequence proximal to a site that allows distinction of specific polynucleotide fragments in a mixed population. This can be accomplished by use of an oligonucleotide (a targeting element) that hybridizes to a sequence next to a polymorphic site (a distinguishing element).

2) Distinction

Once the oligonucleotide is in place, it is enzymatically elongated in a 5'-3' direction. The elongation takes place by incorporation of individual nucleotides, whereby the identity of the base immediately adjacent to the 3'-end of the oligonucleotide (a polymorphic site) establishes a differential in the elongated sequence. This differential can be exploited such that a unique modified nucleotide is provided containing a covalently linked separation element, such as biotin. FIG. 3 shows incorporation of an immobilizable nucleotide.

For example, if "A" is provided with a biotin moiety attached to it, only those fragments having a "T" at the polymorphic site will obtain the separation element on the hybridized oligonucleotide. The oligonucleotides on other fragments will also get elongated but with nucleotides not containing a biotin moiety.

It is preferable that the non-targeted fragments not obtain a separation element. Such incorporation could, for instance, take place if further downstream, i.e. in the direction of enzymatic elongation, a non-targeted fragment were to possess a "T", in which case a biotinylated "A" might be incorporated after the polymorphic site for the 'incorrect' allele. The problem is eliminated by use of terminating nucleotides, such that the elongation of the oligonucleotide stops after the first incorporated nucleotide and no separation element can be attached unless the base immediately adjacent to the 3'-end of the oligonucleotide leads to its incorporation.

A modification of the method allows the use of non-terminating nucleotides. This approach exploits the ability of a polymerase to recognize mismatched oligonucleotides - rather than mismatched individual nucleotides - to accomplish the distinction between targeted and non-targeted fragments: In this case an oligonucleotide is chosen such that it partially overlaps a polymorphic site during hybridization to the fragments, with the mismatch preferentially located at or near the 3'-end of the oligonucleotide, which is the location of enzymatic activity during elongation. Annealing of an oligonucleotide to complementary and mismatched target sequences is shown in FIG. 4.

The oligonucleotide thus only gets elongated if the entire oligonucleotide hybridizes to the fragment (FIG. 5). Conditions can be chosen such that hybridization of a perfectly matched oligonucleotide is highly favored over hybridization of the same oligonucleotide to any site containing a mismatch [3] and if the oligonucleotide-fragment complex does not contain a base-mismatch that prevents the enzyme from binding and initiating the polymerization [4]. If biotinylated nucleotides are present in the reaction, only elongated oligonucleotides bound to targeted fragments will obtain a separation element in the form of multiple incorporated biotins.

3) Separation

In a final step the fragments are separated into fractions that contain the targeted fragment (for example of maternal origin) versus other, non-targeted fragments (for example of paternal origin). This is accomplished by immobilizing the targeted fragment to a solid support which contains a second element with an affinity for the separation element, for instance by immobilizing the biotinylated oligonucleotide-fragment complex to streptavidin-coated magnetic beads, before in a washing step the unbound fraction of the sample is isolated from the beads containing the targeted fraction, allowing for separate analysis of both fractions. Use of a solid support to separate target fragments is shown in FIG. 6.

Automated and High-throughput operation

The invention can be practiced in a fully automated embodiment by use of standard robotic liquid handling and sample preparation systems. In particular, robotic systems are commercially available that utilize magnetic beads to perform the extraction of DNA from a sample in a way that closely resembles the manual operation of such protocols. The adaptation

of the method to those systems and their integration into a fully automated process line is straightforward; it requires no modification of equipment other than programming the system.

Miniaturization and Separation of Genomic DNA Fragments

Miniaturization of the method as well as the separation of long fragments of genomic DNA is desirable in order to examine potentially small samples of tissue, for instance in cancer diagnosis, typing, and prognosis, and to obtain information about polymorphisms located over large contiguous regions. Fragments as large as 1-2 Mbp have been extracted from cells and manipulated for gel electrophoresis. [5].

The method can be performed on a single-molecular level. As an example, individual optically trapped streptavidin-coated beads can be used to capture single or numerous targeted fragments and manipulate them for instance in a microstructure [6]. Targeted fragments can be transported to separate locations such as different chambers of a microstructure for further processing (for instance amplification or sequence analysis [7]) or removal from the microstructure). The original sample is conserved with the exception of the targeted fragments and can be re-used for subsequent extraction of different fragments.

FIG. 7 illustrates repeated events of attachment (twice) and loss (once) of 50,000 base pair long DNA strands to a 1μm bead through hybridization of a biotinylated 16-mer oligonucleotide to the targeted fragment. Motion relative to the fluid exerts a force on the optically trapped bead, which can be measured in displacement on the vertical axis versus time. The oscillating pattern is the result of a back-and-forth motion of the optical trap which generates the displacement signal (see [6] for a detailed explanation). The significant events are changes in the envelope of the pattern, signaling attachment or occasional loss of individual molecules of DNA on the bead.

FIG. 8 illustrates two attachment events of a single DNA of 100,000 base pairs length to a 1 μm optically trapped bead. Losses of fragments are eliminated by ligating the biotinylated oligonucleotide to the targeted fragment; it is easily possible to work with long molecules of DNA for extended times. Direct fluorescent observation was used to confirm the attachment and observe the strand physically being removed from one region to another for storage or further manipulation.

A miniaturized and integrated device is a preferred platform in which the method can be practiced for instance for diagnostic purposes. This embodiment can readily be adapted to

standard methods and devices for miniaturized, inexpensive and integrated genotyping and sequence analysis [8].

Combinations of Terminating and Non-Terminating Nucleotides

It is possible to use combinations of terminating and non-terminating nucleotides, and it is not in all cases necessary that the oligonucleotide binds immediately adjacent to the polymorphic site:

In this example an intervening sequence is present between the binding location of the targeting element and the polymorphic site distinguishing the two alleles:

5' GATTACCAAAAATTC... 3' (SEQ ID NO:1) (allele 1) versus

GATTACCAAAAAGTC... (SEQ ID NO:2) (allele 2)

The two alleles can be distinguished by use of an oligonucleotide binding at the underlined sequence, in which case the heterozygous site, in bold script, is not immediately adjacent to the 3'-end of the oligonucleotide (the polymorphic site is a "T" in allele 1 and a "G" in allele 2) by, for instance, providing

modified, but not necessarily terminating "A" with a separation element attached

non-terminating "T" without a separation element

unmodified, not necessarily terminating "G"

terminating but otherwise unmodified "C"

When the reaction is carried out, only allele 1 will obtain a separation element by which it can be captured.

Other Methods of Performing the Distinction Step

Not only polymerizing reactions as described above but more generally any distinguishing reaction that creates an allele-specific separation element enables the separation of targeted and non-targeted fragments. Many molecular biological as well as chemical methods exist or can be adapted to perform such a selective attachment or removal [9] of a separation element. For example, a population of nucleic acid molecules can be contacted with a targeting element attached to a separation group. The separation group is then removed from the bound targeting element. The remaining immobilizing groups are then immobilized on a substrate, forming an immobilized targeting element-separation group complex. The immobilized targeting element-separation group complex is then removed from the nucleic acid of interest, thereby separating said nucleic acid sequence of interest from said population of nucleic acid molecules.

Binding of the Targeting Element to the Targeted Fragment

a) Initial binding during targeting step

The targeting of polynucleotide fragments with sequence-specific oligonucleotides is straightforward when both are present in single-stranded form. A melting temperature can be calculated for each oligonucleotide-fragment complex below which hybridization occurs. It is possible to adjust the hybridization conditions (mainly temperature and salt / cation concentration) such that only perfectly matched oligonucleotides bind to the fragment. Considerable literature and protocols exist on the polymerase chain reaction (PCR), dye-terminator sequencing reactions as well as mini-sequencing or primer extension reactions, that are of similar nature as the enzymatic distinction reaction in this invention [10,11]. Single stranded DNA can be generated in several ways, for instance by heating and subsequent quenching on ice, NaOH denaturation or physical separation based on biotinylated PCR-primers that get incorporated into only one copy of a PCR product [10,12].

If double-stranded DNA is used as a template, such as genomic or plasmid DNA, the targeted location has to be rendered accessible in order for the oligonucleotide to bind to the fragment. This can be accomplished by heating the sample to a temperature at which the DNA begins to melt and form loops of single-stranded DNA.

Under annealing conditions and typically in an excess of oligonucleotide relative to template, the oligonucleotides will - due to mass action as well as their usually smaller size and thus higher diffusion coefficient - bind to homologous regions before renaturation of the melted fragment strands occurs. Oligonucleotides are also able to enter double-stranded fragments at homologous locations under physiological conditions (37 ° C) [13].

This is relevant since the possibility of cross-hybridization between opposite strands of different alleles can lead to the extraction of a mismatched double-stranded hybrid of two alleles. It is usually undesirable to generate fully single-stranded template DNA due to this reason, although a robust link of the separation element and the targeted fragment - as discussed below - is able to retain the targeted fragment even under harsh denaturation and washing conditions.

Methods and kits have been developed to facilitate the sequence-specific introduction of oligonucleotides into double-stranded targets such as genomic or plasmid DNA [13,14]. A coating of oligonucleotides with DNA-binding proteins such RecA (*E. coli* recombination protein "A") or staphylococcal nuclease speeds up their incorporation several orders of magnitude compared to the introduction of analogous unmodified oligonucleotides at higher concentration and significantly increases the stability of such complexes [15], while still permitting enzymatic elongation of the introduced oligonucleotide [13].

b) Binding during the separation step

It is possible to immobilize or otherwise capture very large molecules and complexes by a single separation element [6,14,16]. If mere hybridization between homologous regions is utilized, the length of the oligonucleotide-separation element has to be chosen of sufficient size to prevent a loss of the fragment during manipulation. For fragments of several hundred to thousand bases size relatively short oligonucleotides (20 bases) are sufficient, whereas longer fragment molecules will require oligonucleotides that bind over larger distances.
It is important to note in this context that under conditions of manipulating fragments relative to the surrounding solution by means of an oligonucleotide-separation element the stability of hybridization is somewhat reduced, since temporary melting due to thermal fluctuations will occur on parts of the sequence that may lead to strand dissociation of a complex that is stable if there is no relative motion between components of the solution.

Another method for increasing the stability of the oligonucleotide-separation element-fragment complex is to provide a targeting element with the separation element already attached

and further stabilize the binding in the distinction reaction. As an example, an oligonucleotide with biotinylated nucleotides incorporated during synthesis is elongated at its 3'-end with regular nucleotides (i.e. not containing biotin) over a significant distance after it has hybridized with the homologous region on the target fragment.

The enzymatic distinction between targeted and non-targeted fragments based on the identity of the targeted polymorphic site is achieved as discussed above before separation is achieved under conditions that facilitate hybridization of greatly elongated oligonucleotides to the targeted fragment and dissociation of short, unextended oligonucleotides from non-targeted fragments. This mode enables the use of oligonucleotides of relatively short length and converts them into tightly binding separation elements once they have been elongated after hybridization to the target fragment.

It is advantageous if a covalently or topologically linked bond is formed (or cleaved) between the separation element and the targeted fragment as a result of the distinguishing reaction. The former can be achieved by providing a reactive group linked to the separation element, so that upon selective incorporation of the separation element the reactive group is irreversibly attached to targeted fragments only. Examples for reactions that can be used for this purpose are described for instance in [17,18,19]. Examples for the formation of topologically linked bonds are described in [20].

Binding of the targeted fragment to the solid support

In the example discussed above, in which a regular oligonucleotide (not containing biotin) is elongated by use of non-terminating biotinylated nucleotides, a particularly strong attachment is formed by multiple binding events between multiple separation elements (i.e. biotins) and solid support (i.e. streptavidin-coated beads). It is significant that the elongation of the oligonucleotide produces numerous separation elements located over a potentially long distance of the targeted fragment. This is shown schematically in FIGs. 5 and 9.

Due to the twisted helical structure of double-stranded DNA, this complex binds to a for instance streptavidin-coated surface in a way that topologically links the targeted fragment to the solid support, provided the distance of the elongated region is significantly greater than the average distance between incorporated biotinylated nucleotides and the pitch of the helix (about 3.4 nm or ten basepairs per turn).

In a related version of the method, topologically improved binding of the targeted fragment to the solid support is achieved by use of multiple targeting and separation elements that simultaneously bind the fragment to a solid support with intervening sequences in between each element pair. It is necessary that such multiple targeting elements co-identify the targeted fragment so as to prevent binding of any of such elements to non-targeted fragments.

In preparation for the separation step it is advantageous to achieve fast on-rates as well as high selectivity and efficiency of binding between targeted fragments and solid support. If small fragments are used, it is sufficient to carry out the binding step by incubation on a rotator at room temperature. In the case of increasingly large fragments, two factors will interfere with the reaction and result in slower and less efficient binding:

a) increasingly large fragments have a significantly reduced diffusion coefficient

b) if only one separation element is present on the fragment, other regions of the same fragment may interfere with the binding step by effectively shielding the separation element from getting into sufficiently close proximity to the solid support to initiate the binding reaction Relative motion between the targeted fragments and the solid support overcomes both problems. This can be achieved by different means, for instance by moving beads used for capturing back and forth through the solution by repeated precipitation and resuspension, or by electrophoretically generated movement.

Non-specific binding to the solid support

Any non-specific binding of non-targeted fragments to the solid support may result in incomplete separation of targeted and non-targeted fragments. Especially single-stranded DNA may readily bind to untreated magnetic beads or other surfaces. The problem is overcome by exposing the surface to a solution containing components that saturate unspecific binding sites on the surface but do not interfere with the specific binding of the separation element [21]. As an example, a blocking buffer "MBSB" is used to suppress unspecific binding to beads (2.8 µm magnetic beads 'Dynabeads M-280 Streptavidin', Dynal A.S., Oslo, Norway, or 1 µm polystyrene beads ('Streptavidin Coated Latex'), Interfacial Dynamics Corporation, Portland, OR) with the result that biotinylated fragments are readily amplified by PCR compared to undetectable levels of product of non-biotinylated fragments on both types of beads (magnetic or polystyrene).

Buffer 'MBSA' is a solution containing 10 mM Tris pH 7.5, 2 mM EDTA, 0.2% Tween-20, 1M NaCl, 5 μg/ml BSA, 1.25 mg/ml 'carnation' dried milk (Nestle), 1 mg/ml glycine. Buffer 'MBSB' is identical to 'MBSB' with the addition of 200 ng/μl sheared salmon sperm DNA (GIBCO BRL), average size ≈ 1000 basepairs, boiled for 3 min. and quenched on ice, and 50 nM each of oligonucleotides of the sequences TTAGTGCTGAACAAGTAGATCAGA (SEQ ID NO:3) and GTATATTCCAAGATCCATTAGCAG (SEQ ID NO:4).

Beads are washed twice in 1 ml "MBSA" by briefly vortexing and precipitating. Precipitation is performed with a particle collection magnet (Polysciences, Warrington, PA) for 1 min. (magnetic beads), or by centrifugation at 13,000 rpm on a table-top centrifuge for 3 min. (polystyrene beads). The beads are then incubated in 100 μl "MBSB" in a fresh tube rotating at RT for 2 hours and stored refrigerated in "MBSB".

Biotinylated and non-biotinylated fragments of identical sequence and 225 basepairs length were generated by PCR amplification of a region in the HLA (human leukocyte associated) locus.

An alternative to prevent contamination with unspecifically extracted, non-targeted fragments is the use of a cleavable linker, which enables the selective release of targeted fragments into solution after separation has been completed [22].

Multiplexing

The method can be performed in a multiplexed fashion by targeting more than one fragment or more than one region on a fragment at once. As an example, this can be accomplished by use of multiple oligonucleotides of different sequence that target different polymorphisms.

If the polymorphisms are all of the same type (for instance all "T"s), all targeted fragments can be extracted with the same type of separation element, in this example a biotinylated "A" (termed "first order multiplexing", shown in FIG. 10). If the polymorphisms are of different type, various separation elements attached to different types of nucleotides can be used to selectively extract the corresponding fragments (termed "second order multiplexing", shown in FIG. 11): For instance, all polymorphisms of type "T" may be targeted by the use of a biotinylated "A" and extracted with streptavidin-coated beads, all polymorphisms of type "C" with fluorescein-modified "G" and beads containing antibodies against "G", and so on. This

embodiment is especially useful if alleles of a sample are to be separated for which the genotype at a certain targeted polymorphic site is unknown.

Generation of a haplotyping library

The method can be used to separate DNA (originating from chromosomal fragments of a sample containing multiple alleles) into fractions that contain the separated alleles only, and overlapping heterozygous regions of different fragments can be used to assemble information on coinherited genomic regions spanning contiguous fragments. Such a library can repeatedly be analyzed at different regions to study polymorphisms that were not classified previously, without the need for further separation of alleles.

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